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FAST TRACKS

Phosphorylation Protects Sperm-Specific Histones H1 and H2B From Proteolysis After Fertilization

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Abstract At intermediate stages of male pronucleus formation, sperm-derived chromatin is composed of hybrid nucleoprotein particles formed by sperm H1 (SpH1), dimers of sperm H2A-H2B (SpH2A-SpH2B), and a subset of maternal cleavage stage (CS) histone variants. At this stage in vivo, the CS histone variants are poly(ADP-ribosylated), while SpH2B and SpH1 are phosphorylated. We have postulated previously that the final steps of sperm chromatin remodeling involve a cysteine-protease (SpH-protease) that degrades sperm histones in a specific manner, leaving the maternal CS histone variants unaffected. More recently we have reported that the protection of CS histones from degradation is determined by the poly(ADP-ribose) moiety of these proteins. Because of the selectivity displayed by the SpH-protease, the coexistence of a subset of SpH together with CS histone variants at intermediate stages of male pronucleus remodeling remains intriguing. Consequently, we have investigated the phosphorylation state of SpH1 and SpH2B in relation to the possible protection of these proteins from proteolytic degradation. Histones H1 and H2B were purified from sperm, phosphorylated in vitro using the recombinant α-subunit of casein kinase 2, and then used as substrates in the standard assay of the SpH-protease. The phosphorylated forms of SpH1 and SpH2B were found to remain unaltered, while the nonphosphorylated forms were degraded. On the basis of this result, we postulate a novel role for the phosphorylation of SpH1 and SpH2B that occurs in vivo after fertilization, namely to protect these histones against degradation at intermediate stages of male chromatin remodeling. J. Cell. Biochem. 76:173–180, 1999.

Key words: protease; male pronucleus; histone phosphorylation; chromatin

After fertilization, the sperm nucleus is transformed into the male pronucleus that fuses with the female pronucleus, reestablishing the diploid zygotic genome. Whole sperm histones are lost from zygote chromatin after amphimixis, suggesting that sperm histones proteolysis is involved in male chromatin remodeling [Poccia and Green, 1992; Imschenetzky et al., 1991, 1996a]. We have identified a nuclear cysteine protease that degrades sperm histones, leaving the maternally derived cleavage stage (CS) histones intact [Imschenetzky et al., 1997]. The protection of CS histones against proteolysis is modulated by the poly(ADP-ribosylation) of the maternally derived set of histone variants [Morin et al., 1999]. In sea urchins, at initial steps of male pronucleus remodeling, sperm histones H1, H2B, and CSH2A are phosphorylated [Green and Poccia, 1985, 1989]. This step is consistently blocked in vivo as well as in vitro by protein kinase inhibitors [Cothreen and Poccia, 1993; Cameron and Poccia, 1994]. Immunobiochemical evidence indicates that at an intermediate step of male pronucleus formation, sperm chromatin is organized into hybrid nucleoprotein particles formed by sperm-specific histones H1 (SpH1) and H2A-H2B (SpH2A-SpH2B), and by a subset of CS histone variants derived from maternal stores [Imschenetzky et al., 1991; 1996a; 1996b]. Therefore, in the context of the selective proteolysis of the complete set of sperm histones by the SpH-protease, these hybrid nucleoprotein particles containing a partial subset of sperm histones together with CS histone variants are a puzzling observation.

In Batracians, the role of nucleoplasmin in the process of the decondensation of sperm chro-

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matin is well documented [Philpott and Leno, 1992]. This pentameric protein promotes the removal of two sperm-specific basic proteins concomitant with the assembly of dimers of the histones H2A-H2B, forming nucleosome cores. Additional evidence indicates that casein kinase 2 (also known as protein kinase CK2) associates and phosphorylates nucleoplasmin, facilitating its accumulation in the nucleus [Vancurova et al., 1995]. Although the presence of nucleoplasmin has not yet been reported in sea urchins, and the protein kinase responsible for the phosphorylation of sperm histones H1 and H2B is unknown, it seemed to us an attractive hypothesis that the phosphorylation state of SpH1 and SpH2B [Green and Poccia, 1985] may determine the stability of these histones at intermediate stages of male chromatin remodeling.

To test this hypothesis, we have isolated sperm-specific H1 and H2B from the sea urchin *Tetrapygus niger*. Both proteins were phosphorylated in vitro with the recombinant α-subunit of casein kinase 2 from *Xenopus laevis* and then assayed as substrates of the SpH-protease. It was found that the phosphorylated forms of these histones are protected from degradation, while the nonphosphorylated forms were readily degraded. These results indicate that the phosphorylation of sperm histone H1 and H2B, which occurs after fertilization, may protect these histones from proteolysis at an intermediate step of male chromatin remodeling.

MATERIALS AND METHODS

Isolation and Purification of Sperm Histone Variants H1 and H2B

Histones were isolated from sperm of sea urchins *Tetrapygus niger*, as described previously [Imschenetzky et al., 1991]. Sperm histone H1 and H2B (SpH1 and SpH2B) were purified by exclusion chromatography on Bio-Gel P-60 according to von Holt et al. [1989]. The peaks containing SpH1 and SpH2B were pooled, freeze-dried, and further purified by high-performance liquid chromatography (HPLC) as follows: A total of 200 µg of the freeze-dried SpH1 and SpH2B fractions was dissolved in 6 M urea-0.1% mercaptoethanol and injected into a µBondapack C18 column (3.9 × 300 mm) (Millipore, Waters Division). HPLC was performed for 90 min at room temperature with a flow rate of 0.5 ml/min. A multistep acetonitrile gradient was applied (solvent A: 0.1% trifluoroacetic acid [TFA] in water; solvent B: 0.1% TFA in 70% acetonitrile). The column was run with solvent A for 10 min; the concentration of solvent B was then increased as follows: 0–50% (for 5 min), 50–100% (for 55 min), and 100–0% (for 10 min). The eluate was monitored at 229 nm, and the fractions containing SpH1, or alternatively SpH2B, were pooled, concentrated, and stored at −20°C. Purified fractions were further analyzed on 18% (w/v) polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE), as described by Laemmli [1970].

SpH-Protease Purification

The SpH-protease was isolated from the chromatin of sea urchin zygotes harvested 5 min after fertilization. Purification was done on a sucrose gradient (10–40% w/v), and followed by exclusion chromatography on Sephadex G-100 (120 mesh), as described previously [Imschenetzky et al., 1997]. The peak containing the activity of the protease was collected, concentrated, and stored at −20°C. No significant loss of protease activity was observed after ≤2 months of storage. ^{14}C-labeled sperm histone H1 was used as substrate to follow the enzyme activity in all steps of purification [Imschenetzky et al., 1997].

Phosphorylation of Sperm Histones H1 and H2B

Histones H1 and H2B were phosphorylated in vitro by the recombinant α-subunit of casein kinase 2 (CK2), as described by Hinrichs et al. [1993]. The enzyme was expressed in *Escherichia coli* BL21 (DE3) using pT7–7 expression vector, and subsequently purified by DEAE-cellulose and phosphocellulose chromatography. For histone phosphorylation, 500 µg of histones were added to 300 µl of a reaction mixture containing 50 mM Hepes pH 7.8, 100 mM KCl, 7 mM $MgCl_2$, 0.5 mM dithiothreitol, and 50 µM [γ-^{32}P]ATP (500–1,000 cpm/pmol). The reaction was started by adding 20 µl containing 0.4 pmol/µl of CK2 and the incubation was carried out at 30°C for the time indicated in each experiment. A total of 10 µl was spotted on Whatman P81 circles of phosphocellulose paper that were then immersed in 75 mM phosphoric acid, washed three times with the same solution, dried, and counted. Values reported were corrected for controls run in the presence of heat-denatured enzyme. All assays were performed in duplicate.

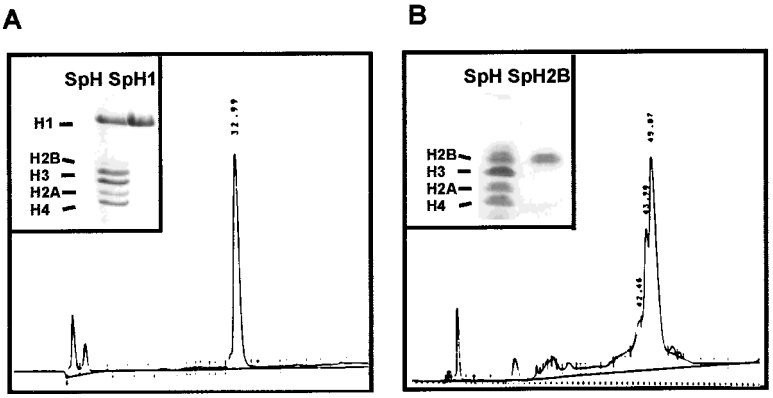

Fig. 1. Reversed-phase high-performance liquid chromatography (HPLC) fractionation of sperm histones. Sperm histones were fractionated by exclusion chromatography on Bio-Gel P-60. Fractions containing SpH1 and SpH2B were pooled, dried, and further purified by HPLC, as described under Materials and Methods. **A:** HPLC elution profile obtained for 200 µg of SpH1. **Inset:** sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of total sperm histones (SpH) and the peak eluted identified as SpH1. **B:** HPLC elution profile obtained for 200 µg of SpH2B. **Inset:** SDS-PAGE of core sperm histones (SpH) and the peak eluted identified as SpH2B.

To assess histone phosphorylation, these proteins were extracted from the reaction mixture with 0.25 N HCl, precipitated with 20% trichloroacetic acid (TCA), washed with 0.7 N HCl/acetone, and then washed three times with cold acetone. The [γ-^{32}P]ATP-labeled-histones were analyzed by electrophoresis in 18% SDS-PAGE, and the radioactivity associated with each histone was evidenced by autoradiography performed as described by Laskey [1980].

Protease Assays and Labeled Protein Substrates

Protease activity was determined by measuring the hydrolysis of labeled protein substrates into TCA. Soluble peptides were determined, as described previously [Imschenetzky et al., 1997]. The nonphosphorylated forms of SpH1 and SpH2B were labeled in vitro with [^{14}C]formaldehyde and used as substrates, as described previously [Imschenetzky et al., 1997]. The labeling of proteins achieved was: [^{14}C]methyl-labeled SpH1 (specific activity 28.000 cpm/µg protein), [^{14}C]methyl-labeled SpH2B (specific activity 23.500 cpm/µg of protein). The phosphorylated forms of SpH1 and SpH2B were obtained, as described above, and assayed as substrates of the protease. The enzymatic labeling of histones achieved was: [γ-^{32}P]ATP-labeled SpH1 (specific activity 6,300 cpm/µg protein), [γ-^{32}P]ATP-labeled SpH2B (spec act 10,900 cpm/µg of protein).

The protease activity is reported as the amount of radioactivity released as TCA-soluble peptides (cpm/µg protein at 37°C). The TCA-insoluble proteins/peptides remaining after digestion were subjected to SDS-PAGE, and the radioactivity associated with each electrophoretic band was detected by fluorography [Chamberlain, 1979]. When phosphorylated histones were assayed as substrates of the protease, the release of [γ-^{32}P]ATP-labeled TCA-soluble peptides was determined. The peptides remaining after incubation with the protease were further analyzed by SDS-PAGE, and the radioactivity associated with each band was determined by autoradiography [Laskey, 1980].

RESULTS

Purification and Phosphorylation of Sperm Histones H1 and H2B

Histones SpH1 and SpH2B were purified by exclusion chromatography on Bio-Gel P-60 followed by HPLC. The HPLC elution profile obtained for SpH1 and SpH2B is shown in Figure 1, as well as the electrophoretic migration, as analyzed by SDS-PAGE. As shown, both SpH1 (Fig. 1A) and SpH2B (Fig. 1B) were purified from other sperm histones by following this procedure. SpH1 is represented by a single variant, whereas histone H2B is present as two variants ($SpH2B_1$ and $SpH2B_2$). The heterogeneity of histone H2B, is consistent with its variability in sperm of other species of sea urchins [von Holt et al., 1989].

Putative casein kinase 2 phosphorylation sites in accordance with the known consensus sequence for this enzyme (S/T)-X-X-(D/E) [Pinna et al., 1990] were examined in the sequences of SpH1 and SpH2B from the sea urchin *Parechinus angulosus* [Strickland et al., 1980, 1977a,b]. Two consensus sites are detected in the SpH1 species; in addition, two less conventional sites in SpH2B. SpH1 and SpH2B variants from *Parechinus angulosus* exhibit a remarkable immunological homology with the homonymous histone variants from the sea urchin *Tetrapygus niger* (unpublished results). Probable phos-

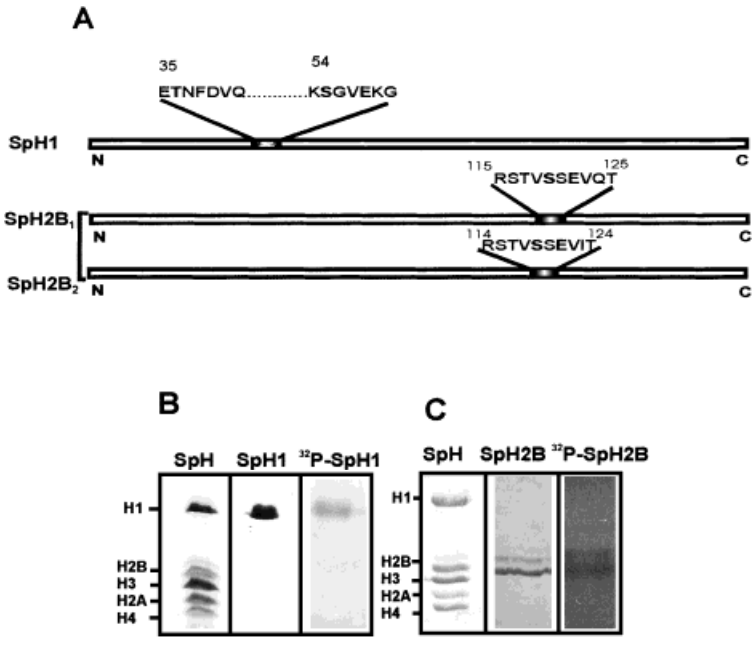

Fig. 2. In vitro phosphorylation of SpH1 and SpH2B by casein kinase 2. **A:** Putative casein kinase 2 phosphorylation target sites of SpH1 and SpH2B from the sea urchin *Parechinus angulosus*. **B,C:** 500 µg of each, SpH1 or SpH2B, were phosphorylated in vitro by the recombinant α-subunit of casein kinase 2, as described under Materials and Methods. The histones obtained after each incubation were extracted with 0.25 M HCl, as described under Materials and Methods, and then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography. **B:** SpH, total sperm histones stained with Coomassie brilliant blue; SpH1, high-performance liquid chromatography (HPLC)-purified SpH1 stained with Coomassie brilliant blue and ^{32}P-SpH1: autoradiography of the phosphorylated form of SpH1. **C:** SpH: total sperm histones stained with Coomassie brilliant blue; SpH2B: HPLC purified SpH2B stained with Coomassie brilliant blue and ^{32}P-SpH2B: autoradiography of the phosphorylated forms of SpH2B.

phorylation target sites for SpH1, $SpH2B_1$, and $SPH2B_2$ variants are shown in Figure 2A.

Purified SpH1 and SpH2B variants were phosphorylated in vitro with casein kinase 2; after 30 min of incubation, the maximal level of phosphorylation was achieved. A single phosphorylated form of H1 (Fig. 2B, left) and two phosphorylated forms corresponding to $SpH2B_1$ and $SpH2B_2$ variants (Fig. 2C, right) were confirmed by autoradiography.

Phosphorylation Protects Sperm Histones H1 and H2B Against Proteolysis

Phosphorylated and native sperm histones were tested as potential substrates of the SpH-protease. As shown, the SpH-protease caused extensive hydrolysis of the native, nonphosphorylated forms of SpH1 (Fig. 3A) and SpH2B (Fig. 3B), whereas the phosphorylated forms of SpH1 (Fig. 3A) and SpH2B (Fig. 3B) remained unaffected. These results were confirmed by the autoradiography of the proteins/peptides remaining after the incubation of these histones in their native and phosphorylated form with the SpH-protease (Fig. 4). It can be seen that the native nonphosphorylated forms of the SpH1 (Fig. 4A, left) and SpH2B (Fig. 4B, left) were almost completely degraded, while the modified forms remain intact (SpH1: Fig. 4A, right, and SpH2B: Fig. 4B, right). No difference was observed in the overall electrophoretic pattern of the peptides obtained after incubation with the protease when unlabeled proteins were used as substrates. The unlabeled peptides derived from the incubation with protease were stained with ammoniacal silver [Morrissey, 1981] (results not shown).

DISCUSSION

It was postulated previously that sperm chromatin remodeling occurs after several interdependent states. The initial step is characterized by a fully condensed conical-shaped sperm pronuclei. The intermediate step is seen as an ovoid or swollen male pronucleus containing a partially decondensed chromatin. At the final step, which coincides with amphimixis, the male pronucleus becomes spherical in shape and contains a fully decondensed chromatin [Cameron et al., 1994].

Concerning histone transitions, sperm histone variants SpH1 and SpH2B initially become phosphorylated [Green and Poccia, 1985]. At the intermediate stage of male pronucleus development, immunobiochemical data derived from our laboratory indicate that SpH1 and SpH2B coexist with maternally derived CS histone variants, forming nucleoprotein particles. At the final stage of male pronucleus formation, the chromatin is composed exclusively of CS histone

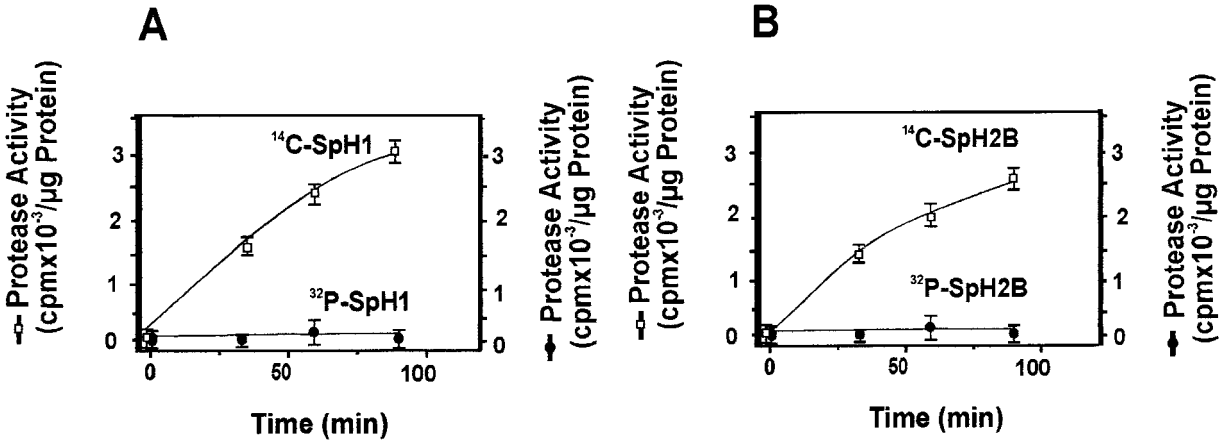

Fig. 3. Phosphorylation protects SpH1 and SpH2B against proteolysis. Protease activity was determined by measuring the hydrolysis of labeled histones into acid-soluble peptides, as described under Materials and Methods. **A:** Time course of the protease activity for the nonphosphorylated (^{14}C-SpH1) and phosphorylated (^{32}P-SpH1) forms of SpH1. **B:** Time course of the protease activity for the nonphosphorylated (^{14}C-SpH2B) and phosphorylated (^{32}P-SpH2B) forms of SpH2B.

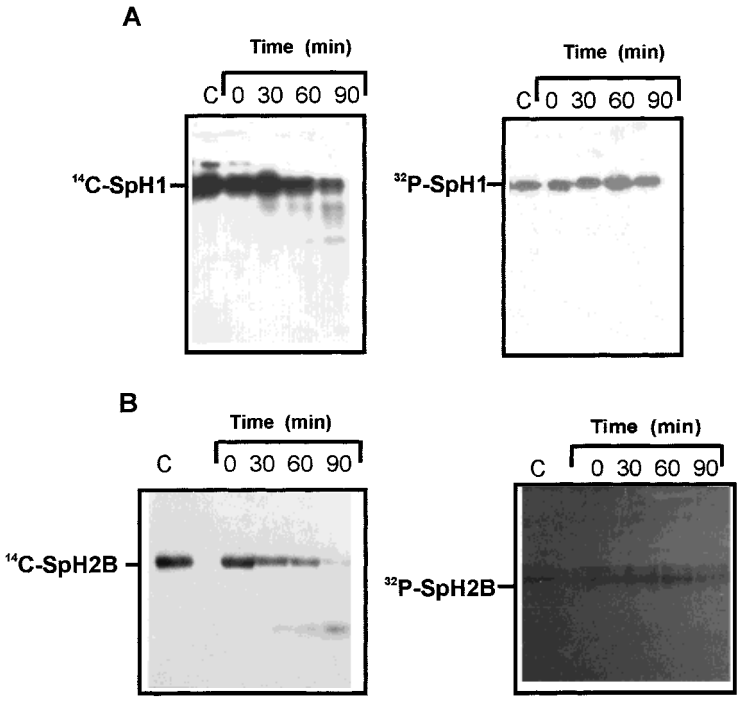

Fig. 4. Phosphorylated forms of SpH1 and SpH2B are protected against proteolysis. Labeled phosphorylated and nonphosphorylated forms of SpH1 and SpH2B were incubated with the SpH-protease, as described under Materials and Methods. The TCA-insoluble proteins/peptides derived from each incubation were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE); the radioactivity associated with each band was detected by fluorography or autoradiography. **A:** Fluorography of protein/peptides derived from ^{14}C-SpH1 (left) and autoradiography of protein/peptides derived from ^{32}P-SpH1 (right). **B:** Fluorography of protein/peptides derived from ^{14}C-SpH2B (left) and autoradiography of protein/peptides derived from ^{32}P-SpH2B (right).

variants; thus, the sperm histones disappear from the nuclei of the zygotes [Imschenetzky et al., 1991, 1996a]. We recently postulated the participation of a nuclear cysteine-protease in male chromatin remodeling. This enzyme degrades sperm histones in a selective manner leaving the maternal CS histone variants unaffected; therefore, it was named SpH-protease [Imschenetzky et al., 1997]. This SpH-protease has been found to be activated in sea urchin zygotes shortly after fertilization, although it is also present in an inactive form in unfertilized eggs [Imschenetzky et al., 1997].

More recently it was demonstrated that the puzzling proteolytic selectivity displayed by the SpH-protease is determined by the poly(ADP-ribose) moiety of the CS histone variants, which protects the modified proteins from degradation [Morin et al., 1999]. Our results described in this report demonstrate that the phosphorylation of SpH1 and SpH2B may protect these sperm histones against degradation by the

nuclear SpH-protease. This protection would explain the coexistence of a partial subset of sperm histones with cleavage-stage variants at intermediate stages of remodeling of male chromatin. Consequently, the SpH-protease postulated to participate in male chromatin remodeling [Imschenetzky et al., 1997] exhibits a unique mechanism of regulating its proteolytic activity, namely, its inhibition by post-translational modification of its substrates, either by its poly-(ADP-ribosylation) [Morin et al., 1999] or by its phosphorylation, as reported herein.

Indirect evidence indicates that male pronucleus decondensation is blocked in vivo and in vitro by the kinase inhibitors 6-dimethylaminopurine (6-DMAP) and staurosporine. In vivo 6-DMAP does not alter the normal pattern of SpH1 and SpH2B phosphorylation, but it affects microtubule growth and blocks pronuclear migration [Poccia et al., 1990; Dufresnee et al., 1991]. Staurosporine inhibits phosphokinase C, G kinase, A kinase, and perhaps other kinases as well [Abe et al., 1991; Crissman et al., 1991], but it does not affect pronuclei migration.

More recently, protein kinase C has been involved in lamin B phosphorylation, thus participating in the disassembly of sperm nuclear envelope at fertilization [Collas et al., 1997]. This enzyme is activated within 120 s after insemination, is Ca^{2+} dependent, and clusters in the cortical zone of the zygotes [Barry et al., 1997]. Otherwise, the phosphorylation target sites described in vivo involve serine/threonine residues located in the N-terminal region of SpH2B variants and at N-terminal and C-terminal regions of SpH1 [Green and Poccia, 1985].

It has been postulated that these phosphorylation target sequences correspond to SPKK motifs clustered in N-terminal regions of SpH1 and SpH2B variants that become reversibly phosphorylated during spermatogenesis [Green et al., 1993]. The phosphorylation of SpH1 and SpH2B weakens the binding capacity of these histones to linker DNA, inducing chromatin relaxation [Green et al., 1993]. However, no definitive conclusions can be derived thus far concerning the phosphorylation of SpH1 and SpH2B that occurs during male pronucleus formation. Evidence concerning the level of phosphorylation of SpH2B variants and SpH1, as well as the precise sequences modified in vivo, is still lacking and should not necessarily be homologued to the reversible phosphorylation that occurs during spermatogenesis. Alternatively, it was reported that casein kinase 2 associates with nucleoplasmin, the chaperone-protein that interacts with H2A-H2B in nucleosomes remodeling [Philpot and Leno, 1992]. Even if the putative serine/treonine phosphorylation target sites deduced in this report (Fig. 2) do not appear to be in close agreement with the location of the phosphorylation sites previously described [Green and Poccia, 1985], this kinase seems to be an interesting candidate in sperm histones phosphorylation. As reported, the CK2 β-regulatory subunit may establish promiscuous interactions with other protein kinases and it has been postulated that casein kinase 2 may act as a "wild card" by interacting with several partners, thus regulating a variety of catalytic activities [Allende and Allende, 1998].

Finally, it was recently described that cyclin-dependent kinase 2 (Cdk2) localizes in male pronucleus during the first few minutes after fertilization [Moreau et al., 1998]. It is well known that Cdk2 is a member of a family of kinases that associate with cyclins that regulate the progression through the cell cycle [reviewed by Nigg, 1995]. Cdk2 association with its potential partners cyclins A and E drives somatic cells' DNA replication–related events [reviewed by Sherr, 1996]. By contrast, in early embryonic cell cycles of sea urchins, the activity of Cdk2 was suggested to regulate the inhibition of DNA re-replicating events [Moreau et al., 1998]. Thus, the location of Cdk2 in male pronucleus long before the initiation of the first zygotic S phase appears as a puzzling observation. Cyclin A was not clearly observed in chromatin before 10 min postfertilization; afterward, it becomes clearly localized in the chromatin of zygotes. Unfortunately, since the data supporting this observation are not reported, it is unclear whether cyclin A localization corresponds to male chromatin or female chromatin, or both. Immunoprecipitated cyclin A from sea urchin zygotes displayed associated H1 kinase activity that increases along the cell cycle, raising its activity very early after insemination, as well as at the end of the first S phase. On the basis of this information, the potential

participation of Cdk2/cyclinA/histone H1 kinase association either directly, or as part of the phosphorylation cascade, in male chromatin remodeling seems worthwhile to consider, if only speculatively for the present. Consequently, the question concerning the kinases responsible for the phosphorylation of SpH1 and SpH2B variants after fertilization remains open. Independent of the protein kinases that phosphorylate sperm histones after fertilization, it seems clear from the results described that this post-translational modification represents a specific manner to protect sperm histones from proteolysis at intermediate stages of sperm chromatin remodeling.

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